Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium as a substitute for trophectoderm biopsy

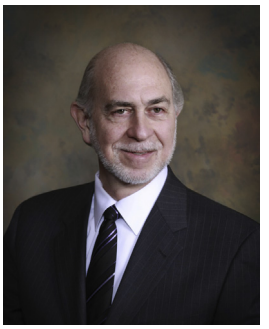
Carmen Rubio, Ph.D.,[a,b] Catherine Racowsky, Ph.D.,[c] David H. Barad, M.D., M.S.,[d,e] Richard T. Scott Jr., M.D., H.C.L.D.,[f,g] and Carlos Simon, M.D., Ph.D.[h,i]

[a] Igenomix, Research and Development and [b] Igenomix Foundation/INCLIVA, Reproductive Genetics, Valencia, Spain, [c] Department of Obstetrics, Gynecology and Reproductive Medicine, Hôpital Foch, Suresnes, France, [d] The Center for Human Reproduction and [e] The Foundation for Reproductive Medicine, New York, New York, [f] IVIRMA New Jersey, Basking Ridge, New Jersey, [g] Sidney Kimmel Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania, [h] Department of Obstetrics and Gynecology, Valencia University and INCLIVA, Valencia, Spain, and [i] Department of Obstetrics and Gynecology, BIDMC Harvard University, Boston, Massachusetts

Disclaimer: Authors for "fertile battles" are chosen to represent the full breadth of opinions. Individual authors, even within one side of the debate, do not necessarily agree with all viewpoints expressed.

PRO: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will substitute for trophectoderm biopsy

Pro 1. Carmen Rubio, Ph.D.



CON: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will not substitute for trophectoderm biopsy

Con 1. David H. Barad, M.D., M.S.

How reliable is embryonic cell-free deoxyribonucleic acid (cfDNA) versus trophectoderm DNA (teDNA) as a representative of embryo chromosomal constitution?

Since the first study that identified embryonic cfDNA in spent blastocyst media (1), its potential as a new noninvasive preimplantation genetic test for aneuploidy (niPGT-A) to assess embryo chromosome copy number has become evident. Since then, several studies have explored the concordance between the copy number of this cfDNA and amplified DNA obtained from polar bodies (2), trophectoderm biopsies (teBxs) (3–9), and whole blastocysts (3, 7, 10, 11).

The reported informativity rates of cfDNA in terms of successful amplification and interpretable results using next-generation sequencing (NGS) ranged between 80% and 100%. The concordance rates of cfDNA with DNA from teBxs

How reliable is embryo cfDNA versus teDNA as a representative of embryo chromosomal constitution?

Both cfDNA and teDNA reliably and consistently help identify euploid blastocysts with a low false-negative rate. However, consistency is different from validity, which is the extent to which a system measures what it claims to measure. The absence of validity can have several causes, including improper sampling or biologic factors that, at the blastocyst stage, make any testing of embryo ploidy inappropriate. The lack of validity of cfDNA and teDNA tests can lead to false-positive diagnoses and the loss of euploid pregnancies.

Preimplantation genetic testing for aneuploidy in its earliest form was discredited as being harmful to embryos and inaccurate (44, 45). Switching embryo biopsy stage from cleavage to blastocyst stage was predicated for improving embryo sampling at a stage when mosaicism was thought to be

You can discuss this article with its authors and other readers at https://www.fertstertdialog.com/posts/32571
C.R. has nothing to disclose. C.R. has nothing to disclose. D.H.B. has nothing to disclose. R.T.S. has nothing to disclose. C.S. is founder and head of the scientific advisory board of Igenomix.
Reprint requests: Carlos Simon, M.D., Ph.D., Igenomix SL, Paterna, Valencia, Spain (E-mail: carlos.simon@uv.es).
https://doi.org/10.1016/j.fertnstert.2021.02.045

PRO: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will substitute for trophectoderm biopsy *(continued)*

varied widely, up to 89.1% for thawed, biopsied blastocysts (7). Interestingly, the cfDNA concordance rate with the DNA of whole blastocysts reached 93.8% (7). This result strongly suggested that embryo cfDNA is robust and can be more representative of embryo chromosomal status than teBxs (7). Further, our recent study (9) obtained concordance rates of cfDNA similar to those of teBx and inner cell mass (ICM) biopsy (87.5% and 84.5%, respectively).

Is embryo cfDNA secretion related to embryo chromosomal self-correction and/or apoptosis?

Apoptosis during preimplantation development has been proposed as a potential mechanism for embryo DNA shedding because of the embryo's rapid, dynamic transformation during late preimplantation development (12). Apoptotic DNA is compromised because of the degradation processes and, therefore, does not provide robust diagnostic information. However, cfDNA exhibits high concordance with teBxs and whole blastocysts. Further, in cases of low-quality blastocysts (e.g., aneuploids), high degrees of apoptosis are expected to lead to shedding of high quantities of DNA. However, this is not the case; the informativity and concordance rates in moderate- or low-quality blastocysts are similar to those of good-quality blastocysts (13). In a recent study, we obtained similar concordance rates of cfDNA with teBx and ICM biopsy (87.5% and 84.5%, respectively) (9), in addition to similar DNA quantity in media from euploid and aneuploid blastocysts, as observed previously (4). Taken together, these findings instead supported the hypothesis that cfDNA secretion is a physiologic phenomenon for each embryo, independent of their quality and ploidy status and not related to self-correction by apoptotic events.

What are the reasons that the noninvasive model will prevail or not over the invasive model?

Noninvasive tests are the ultimate goal in medicine, and indeed, noninvasive prenatal testing has changed the practice of prenatal medicine. In in vitro fertilization (IVF) programs, incorporating preimplantation genetic test for aneuploidy (PGT-A) improves ongoing pregnancy rates per transfer and shortens the time to pregnancy while decreasing miscarriage rates in couples experiencing infertility (14, 15). However, the current practice for PGT-A is invasive, relying on embryo manipulation (teBX) that requires dedicated equipment and highly trained personnel and, therefore, encompasses substantial commitment in terms of time and cost. Moreover, recent studies have associated teBx with a significant increase in pre-eclampsia (16) and hypertensive disorders (17).

An efficient and reliable noninvasive approach could offer the benefits of invasive PGT-A without the accompa-

CON: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will not substitute for trophectoderm biopsy *(continued)*

less prevalent and more genetic material could be safely obtained. In NGS, using a greatly improved diagnostic tool that allowed for the diagnosis of multiple cell lineages, mosaicism became a surprisingly common finding in biopsies. Moreover, mosaic embryos were shown to have an unexpectedly high potential to achieve viable term pregnancies (34, 35, 46, 47), albeit some investigators who claimed a lower rate than that of embryos with euploid biopsy results (47). Considering the currently available data, the ability of a single teBx to reliably reflect the ultimate chromosomal fate of an embryo to improve IVF cycle outcomes must, therefore, be questioned on theoretical (48, 49) and empirical grounds (34, 43, 46, 50).

Cell-free DNA testing was introduced to eliminate the presumed damage to embryos caused by teBx and provide a more comprehensive assessment of an embryo's whole genome than that offered by sampling a few cells (10). Although some studies have found concordance between cfDNA and teBx (9), others have reported significant discordance between teDNA and the sampling of whole-embryo DNA (10), mostly attributed to maternal contamination (in cfDNA) and embryonic mosaicism in general. By far, the best correlation has been reported by Huang et al. (7), with a zero false-negative rate and positive predictive value and specificity that was better with cfDNA than that with traditional teDNA.

The investigators attributed the superiority of their results over those of practically all other studies published so far to the careful removal of cumulus cells before intracytoplasmic sperm injection (i.e., decreasing maternal contamination) and disregarding consideration of all observed mosaicisms $<$60% of the sampled DNA. Although this threshold unquestionably lowered the risk of FPRs, it did not eliminate them; cfDNA and teDNA, indeed, still produced significant FPRs when compared with whole-embryo DNA (20% and 50%, respectively), confirming that with both the methods, there remains a significant risk of discarding potentially viable euploid embryos (7).

Is embryo cfDNA secretion related to embryo chromosomal self-correction and/or apoptosis?

Mosaic embryos have been observed to have a greater rate of cell proliferation and cell death compared with euploid embryos. These observations have led to the suggestion that aneuploid cells selectively leave the embryo as a mechanism of correction of aneuploidy (51). Mouse embryos eliminate aneuploid cells of fetal lineage by autophagy and apoptosis (38, 52), with a similar mechanism more recently also demonstrated in human embryos and gastruloids (53). In mouse and human embryos, the elimination of aneuploid cells was more prevalent in embryonic cell lineage (ICM) than in extraembryonic lineage (TE) (38, 53).

The net result of the selective elimination of aneuploid cells from the blastocyst is the enrichment of the spent media

PRO: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will substitute for trophectoderm biopsy *(continued)*

nying technical and financial burdens. Further, the noninvasive approach could be offered as a prioritization model, in which the testing of cfDNA in culture media, in combination with morphology, could enable "ranking" of viable blastocysts, most likely resulting in a healthy baby. This model can promote blastocyst prioritization so that providers and couples can select the best blastocyst for the first embryo transfer and reserve the others (rather than discard) for subsequent transfers.

With advances in scientific and technical knowledge, research on biomedical microelectromechanical systems is also developing toward lab-on-a-chip devices. The niPGT-A approach might lead to a part of IVF development moving toward automation because the additional steps required to remove cumulus cell contamination and collect a spent culture medium can be easily automated.

What additional evidence is needed to support the pro?

Several publications have provided evidence for the fact that embryonic cfDNA of blastocysts cultured under different conditions yields blastocyst concordance rates similar to those of teBxs (9). However, a strong objection is that an expanded culture till day 6 is required to obtain sufficient cfDNA quantity for robust results (6, 18). Accumulated evidence has indicated using aneuploidy testing that vitrified day-6 blastocysts have ongoing pregnancy rates similar to those of day-5 blastocysts (19). Tiegs et al. (20) showed no differences in sustained implantation rates between day-5 and day-6 single embryo transfers in PGT-A cases, with deferred embryo transfer. This was confirmed by a meta-analysis that concluded using PGT-A that implantation, clinical pregnancy, live birth, and ongoing pregnancy rates were similar for euploid day-5 and day-6 blastocyst transfers (21). These results align with those of previous reports that used PGT-A, with similar outcomes for day-5 or day-6 euploid transfers (22–25). A recent study evaluating the effect of the day of cryopreservation showed that in frozen transfers of euploid embryos assessed by PGT-A, the day of cryopreservation did not significantly affect live birth rates (26).

The remaining question is the potential for this technology to improve its reproductive outcome compared with that of standard IVF. Class I evidence should be obtained from randomized trials comparing the efficacy of niPGT-A with that of current IVF practice without PGT-A for different patient age ranges. In addition, nonselection studies can help reveal the reproductive potential of different patterns of results obtained after cfDNA sequencing. Such data can help inform a scoring system for each pattern according to its capability of resulting in a healthy live birth so as to facilitate its use in clinics.

CON: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will not substitute for trophectoderm biopsy *(continued)*

with cfDNA derived from these aneuploid cells, further increasing the risk of a false-positive diagnosis. One can infer that because aneuploid cells are differentially cast off, ICM becomes progressively more euploid, whereas this process affects TE only to a minor degree. Thus, progressive apoptosis of aneuploid cells is a mechanism of embryo self-correction. Blastocyst-stage embryos, therefore, are chromosomally still "in flux," with their ultimate genetic identity yet to be determined. Chromosomal testing at the blastocyst stage, therefore, appears nonsensical.

What are the reasons that the noninvasive model will prevail or not over the invasive model?

Noninvasive diagnosis is obviously preferred over invasive diagnosis, although only if validated IVF cycle outcomes are similar. The results of all forms of PGT-A between testing laboratories, however, greatly vary (54), suggesting that technical differences between laboratories and the performance of embryo biopsies are important. Because any biopsy has the potential to damage embryos and requires special skills (55), embryologists may prefer a reliable cfDNA diagnostic procedure. Neither teBx nor cell-free biopsy (cfBx), however, addresses the previously noted biological reality that an embryo at the blastocyst stage has not yet reached a steady state in terms of its chromosomal integrity. Neither teBx nor cfBx have so far demonstrated sufficient specificity to reliably eliminate only aneuploid embryos from transfer.

What additional evidence is needed to support the con?

Any diagnostic test needs to have proven sensitivity and specificity before it can be incorporated into daily practice. Both teBx and cfBx have acceptable specificity for the diagnoses of euploidy. But when it comes to the diagnosis of aneuploidy, both the procedures have unfortunately remained inaccurate, best documented by the birth of hundreds of healthy infants following the transfer of embryos previously labeled as "untransferable" based on PGT-A (34, 35, 46).

Published data have suggested that most self-correction occurs quickly between days 5 and 8 of development (38, 52, 53). A PGT-A using cfDNA, therefore, can become more accurate if we learn to extend embryo culture beyond days 5–6 to days 7–8 after fertilization. Cell-free biopsy with the use of spent media from embryos on days 7–8 after fertilization might be able to allow a more reliable assessment of embryo ploidy. However, some embryos might not survive in an extended culture, and transfer might then occur after the optimal window for implantation.

It is astonishing that several IVF centers have already started offering cfBx as a routine clinical practice. This can lead to truly catastrophic consequences, as demonstrated by a recent class action suit in Australia (56). Given the lack of

PRO: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will substitute for trophectoderm biopsy *(continued)*

Pro 2. Catherine Racowsky, Ph.D.

How reliable is embryo cfDNA versus teDNA as a representative of embryo chromosomal constitution?

Following the first report of successful live births after the transfer of embryos selected based on an analysis of cfDNA in a culture medium (10), numerous studies have shown the high reliability of detection and amplification of cfDNA for niPGT-A (27). However, the assessment of copy number variation may be hindered in some cfDNA samples because of noisy profiles (6, 7, 13) and/or contamination due to either the culture medium's constituents (4, 28, 29) or cumulus cells (2, 4, 30). Although typically, >95% of teBxs provide informative results (31), NGS studies of cells obtained from disaggregated blastocysts indicated that approximately 50% of embryos may exhibit some level of mosaicism either among trophectoderm (TE) cells and/or between the ICM and TE lineage (32). Because only a few TE cells were analyzed, mosaicism inherently confounded the diagnostic accuracy of invasive PGT-A with teBx (iPGT-A) (33), as highlighted by numerous reports on healthy births resulting from the transfer of embryos that were classified as "abnormal" based on iPGT-A (34, 35).

Because of the potential for genetic discordance between ICM and TE, the evaluation of the relative reliability of niPGT-A compared with that of iPGT-A for determining the chromosomal constitution of ICM should use the corresponding embryo or ICM as a control gold standard. When data from 4 such available studies assessing cfDNA in a spent culture medium with or without blastocyst fluid (BF) were combined (7, 29, 36, 37; Supplemental Table 1 [available online]), the false-positive rate (FPR) was significantly improved for niPGT-A versus that for iPGT-A ($P = .042$) (Table 1). When only 3 studies in which the medium was collected after blastocyst culture (day 5 or 6 or day 6 or 7) (29, 36, 37) were analyzed, the performance of niPGT-A was superior to that of iPGT-A in terms of FPR ($P = .008$), positive predictive value ($P = .034$), and concordance for both embryo ploidy ($P = .039$) and chromosome copy number variation ($P = .024$) (Table 2). These data indicated that niPGT-A was less prone to errors associated with embryo mosaicism and was more reliable than iPGT-A, a conclusion consistent with the 3-

CON: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will not substitute for trophectoderm biopsy *(continued)*

proven efficacy to improve clinical pregnancy and live birth rates (43), one is, indeed, forced to ask what, under current circumstances, the potential benefits to infertile couples are from any form of PGT-A, whether by teBx or cfBx.

Con 2. Richard T. Scott, Jr., M.D., H.C.L.D.

The desire to enhance implantation rates and reduce clinical losses and ongoing aneuploid gestations has led to steadily increasing use of PGT-A in clinical practice. Evolving analytical platforms, more rigorous validation, and the ability to vitrify embryos with minimal risk have empowered highly successful paradigms for screening (57). A reduced number of transfer order, principally a single embryo transfer, is commonly used by patients choosing PGT-A, which greatly enhances safety without compromising its outcomes (58).

Although the current paradigms are excellent, they still require teBx and precise loading of the biopsied cells into polymerase chain reaction tubes, of which the latter actually requires the greatest technical prowess. Class I data exist that demonstrate that this can be done safely, but it requires training and validation (59).

In 2013, when Palini et al. (60) described the fact that embryonic DNA exists in the blastocoel fluid, many investigators saw the potential to perform PGT-A in a simpler, less invasive way (5, 31). Although an attractive concept, the path to niPGT-A has not been simple and is largely associated with technical challenges and limited clinical trials. There is no doubt that there are many barriers to successful niPGT-A.

The reality that the biology of embryo development may make it impossible for niPGT-A to provide the same clinical value as conventional teBx-based PGT-A is the most concerning. There are data that indicate that embryos may preferentially eliminate chromosomally abnormal cells by apoptosis, which can lead to their DNA being released into the blastocoel or spent culture media (38). Thus, the DNA in these specimens cannot be reflective of the reproductive potential of the embryo itself. Stated simply, niPGT-A performed on spent culture media might provide a perfectly correct analytical result by the detection of aneuploidy but may have little or no relevance to the genetic composition of the remaining embryo and its ultimate reproductive potential.

Another significant biologic barrier relates to the fact that spent media of blastocysts with the highest reproductive potential commonly fails to amplify. In fact, in a recent blinded nonselection study evaluating the predictive value of niPGT-

PRO: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will substitute for trophectoderm biopsy *(continued)*

TABLE 1

Comparison of the performance of niPGT-A and iPGT-A for PGT-A, regardless of the days of culture before sample collection.

Performance characteristic	niPGT-A (n = 139)	iPGT-A (n = 138)	*P* value
FPR	24.4% (10/41)	46.5% (20/43)	.042
FNR	3.1% (3/98)	2.1% (2/95)	1.000
PPV	90.5% (95/105)	82.3% (93/113)	.115
NPV	91.9% (34/37)	94.1% (32/34)	1.000
Sensitivity	96.9% (95/98)	97.9% (93/95)	1.000
Specificity	75.6% (31/41)	60.5% (26/43)	.165
Concordance for embryo ploidy	90.6% (126/139)	84.1% (116/138)	.107
Concordance for whole chromosome CN	71.9% (100/139)	67.4% (93/138)	.233

Note: The comparison includes all 4 studies assessing the performance characteristics of niPGT-A and iPGT-A on whole embryo from samples (spent medium with or without BF) after culture from day 3 to 5, day 5 to 6, or day 6 to 7. *P* values were derived using the chi-square and Fisher's exact tests, with $P < .05$ considered statistically significant. BF = blastocyst fluid; CN = copy number; FNR = false-negative rate; FPR = false-positive rate; iPGT-A = invasive preimplantation genetic test for aneuploidy; niPGT-A = noninvasive preimplantation genetic test for aneuploidy; NPV = negative predictive value; PPV = positive predictive value; PGT-A = preimplantation genetic testing for aneuploidy.

Rubio. To invade or not to invade?. Fertil Steril 2021.

gestation) for transferred embryos classified as euploid based on both niPGT-A and iPGT-A versus those classified as euploid based on iPGT-A but classified as aneuploid based on niPGT-A (6).

Is embryo cfDNA secretion related to embryo chromosomal self-correction and/or apoptosis?

Cell-free DNA secretion is related to ploidy self-correction; this is supported by the fact that clinical and ongoing

TABLE 2

Comparison of the performance of niPGT-A and iPGT-A for PGT-A using samples collected following blastocyst culture.

Performance characteristic	niPGT-A (n = 102)	iPGT-A (n = 100)	*P* value
FPR	21.7% (5/23)	62.5% (15/24)	.008
FNR	1.3% (1/79)	0.0% (0/76)	1.000
PPV	94.0% (78/83)	83.5% (76/91)	.034
NPV	95.5% (21/22)	100.0% (18/18)	1.000
Sensitivity	98.7% (78/79)	100.0% (76/76)	1.000
Specificity	78.3% (18/23)	50.0% (12/24)	.069
Concordance for embryo ploidy	94.1% (96/102)	85.0% (85/100)	.039
Concordance for whole chromosome CN	81.4% (83/102)	67.0% (67/100)	.024

Note: The comparison includes 3 studies assessing the performance characteristics of niPGT-A and iPGT-A on whole embryo after the collection of samples (spent medium with or without BF) at the blastocyst stage (culture day 5 to 6 or day 6 to 7). *P* values were derived using the chi-square and Fisher's exact tests, with $P < .05$ considered statistically significant. BF = blastocyst fluid; CN = copy number; FNR = false-negative rate; FPR = false-positive rate; iPGT-A = invasive preimplantation genetic test for aneuploidy; niPGT-A = noninvasive preimplantation genetic test for aneuploidy; NPV = negative predictive value; PPV = positive predictive value; PGT-A = preimplantation genetic testing for aneuploidy.

Rubio. To invade or not to invade?. Fertil Steril 2021.

CON: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will not substitute for trophectoderm biopsy *(continued)*

A for sustained implantation rate (the gold standard for any PGT study), the implantation rates were higher in the failed amplification group than those in the euploid group (61). This suggested that the highest-quality embryos likely shed fewer apoptotic cells and, thus, have less DNA available in the spent media, which compromises the analytical process. A test in which embryos with the best prognosis are classified as having "no result" is not clinically useful because it cannot be used to safely direct selection. The no-result group still contains aneuploid embryos that put patients at a risk of adverse outcomes.

It is worth commenting on the reports that have demonstrated that the average DNA content in spent media is sufficient to provide a reliable analytical result (62). The reality is that the mean level may be sufficient, but it is not clinically relevant. Clinicians and embryologists need reliable results for all evaluable embryos; thus, a useful technique requires that $\geq$98% of samples have sufficient DNA to provide an analytical result. Such a high threshold is chosen because the level of efficiency has already been clinically shown using trophectoderm-based PGT-A, which is already being performed safely in many programs around the world. There is no reason to compromise and accept a poorer performing approach.

The issues with the reliability of the analytical platforms with these very small quantities of DNA remain to be refined and validated. Many technical questions remain to be solved using large prospective studies. What type of amplification should be used? Most investigators have used whole-genome amplification, but this technology may be quite unreliable because of very limited quantity of starting material. Is it necessary to sample the blastocoel fluid and spent media? What is the best timing for sample collection? Obviously, not all embryos will be in the developmental stage at the same time. Does that mean that embryologists have to follow these embryos throughout days 5 through 7 and collect and load these samples at multiple times for each patient? That would be extremely challenging logistically.

The timings of media change and duration of exposure of the embryo are also associated with many challenges. Some investigators have suggested that the best samples are obtained from media used to culture embryos from days 4 to 6 (7). Of course, this will mean extra manipulation in the laboratory on day 4 and the necessity to continue culture to day 6 even if the optimal time to vitrify the embryo is on day 5. How much volume should embryos be cultured in? Is it necessary to sample the blastocoel? Large, well–designed, prospective studies need to be conducted to compel embryologists and clinicians about the safety, efficacy, and optimization of these techniques. Currently, no such large comparative studies are available.

In conclusion, it is worth commenting on the types of data that should be published before the clinical implementa-

PRO: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will substitute for trophectoderm biopsy *(continued)*

pregnancy rates were higher following the transfer of embryos classified as euploid based on iPGT-A but without cfDNA detected in their BF compared with the transfer of those with BF cfDNA detected (38) and that in a murine model (39), the frequency of apoptosis in ICM of aneuploid cell clones was increased compared with that of ICM of euploid clones (41.4% vs. 19.5%, respectively, $P<.0001$); both the frequencies were much higher than those in their TE counterparts (3.3% vs. 0.6% for euploid and aneuploid clones, respectively, $P<.001$).

The apoptotic origin of cfDNA was supported by the finding that BF cfDNA comprises 2 peaks, a dominant peak of 160–220 base pairs and another of 300–400 base pairs (40). Of note, the length of the dominant peak is similar to that of circulating cfDNA originating from apoptotic placental trophoblast cells (41, 42).

What are the reasons that the noninvasive model will prevail or not over the invasive model?

niPGT-A will prevail over iPGT-A because of the following reasons: available evidence has indicated the performance superiority of niPGT-A over that of iPGT-A (Table 2) and, therefore, greater reliability of niPGT-A for the determination of embryo karyotype; 1 randomized controlled trial (RCT) evaluated the efficacy of iPGT-A compared with morphological selection, revealing a limited and age-dependent benefit, with implantation rates being increased only in women aged 35–40 years and no benefit in women aged <35 years (women aged >40 years were not included in the trial) (43); and the practical limitations of niPGT-A include the fact that niPGT-A effectively addresses the potential for sample loss in iPGT-A because of the exclusion of poor-quality embryos not suitable for biopsy and the possibility of embryo damage during the biopsy itself; there is no need for embryo biopsy training; and medium collection is easier to standardize.

What additional evidence is needed to support the pro?

More robust data with larger sample sizes are needed to confirm the current data that niPGT-A is less prone to errors associated with embryo mosaicism and is more reliable than iPGT-A for determining embryo ploidy. Technological improvements using single-cell genomics and single-cell multiomics sequencing to detect maternal contamination will lead to advances in this field. The standardization of protocols, including medium collection, technological platforms, amplification, and screening algorithms, is required, including establishing standard mosaicism threshold levels to minimize false mosaic calls caused by noisy profiles (7), indicating that these thresholds likely differ for media

CON: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will not substitute for trophectoderm biopsy *(continued)*

tion of niPGT-A. Studies correlating niPGT-A results with those of traditional PGT-A are not sufficient. This is especially true when most of the PGT-A analytical platforms have not been fully validated. Research that compares their results to those of PGT-A of ICM or multiple teBxs are interesting and will provide a valuable insight into the biology of human embryonic development; however, they are simply inadequate to justify the clinical application of niPGT-A.

At a minimum, a prospective blinded nonselection study demonstrating the predictive value of both euploid and aneuploid results should be published. This should be followed by RCTs that show that the enhanced selection actually improves the sustained implantation rates. The latter is critical because most of the best embryos are unlikely to provide a result and may lead to patients becoming cautious and assisted reproduction technology team members deprioritizing them. It is extremely important that these studies be done. That is true whether or not niPGT-A is used by itself or as an adjunct to teBx-based PGT-A.

It seems likely that at some point in the future, niPGT-A will be an important clinical tool. A long and complex journey will be required before its clinical implementation. The adverse consequences of early implementation are more than just a theoretical concern—they are already a reality (63).

PRO: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will substitute for trophectoderm biopsy *(continued)*

collected from day 3 or 4 to day 5 versus that collected from day 5 to day 6. With such standardizations, a multicenter RCT that assesses miscarriage rates, prenatal diagnostic screening, live birth rates, and, ideally, long-term follow-up is needed to confirm the accuracy and safety of niPGT-A. If the RCT data favors niPGT-A, artificial intelligence based on machine learning can be used to integrate this technology with conventional morphologic grades as well as time-lapse and patient information to further advance the evaluation of the implantation potential of each embryo.

The authors thank Lei Huang, Ph.D., for her assistance in determining some of the values in Table 1.

CON: Noninvasive preimplantation genetic testing for aneuploidy in spent culture medium will not substitute for trophectoderm biopsy *(continued)*

REFERENCES

1. Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. Fertil Steril 2016;106:1312–8.
2. Feichtinger M, Vaccari E, Carli L, Wallner E, Mädel U, Figl K, et al. Non-invasive preimplantation genetic screening using array comparative genomic hybridization on spent culture media: a proof-of-concept pilot study. Reprod Biomed Online 2017;34:583–9.
3. Ho JR, Arrach N, Rhodes-Long K, Ahmady A, Ingles S, Chung K, et al. Pushing the limits of detection: investigation of cell-free DNA for aneuploidy screening in embryos. Fertil Steril 2018;110:467–75.
4. Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, et al. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. Hum Reprod 2018;33:745–56.
5. Yeung QSY, Zhang YX, Chung JPW, Lui WT, Kwok YKY, Gui B, et al. A prospective study of non-invasive preimplantation genetic testing for aneuploidies (NiPGT-A) using next-generation sequencing (NGS) on spent culture media (SCM). J Assist Reprod Genet 2019;36:1609–21.
6. Rubio C, Rienzi L, Navarro-Sánchez L, Cimadomo D, García-Pascual CM, Albricci L, et al. Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: concordance rate and clinical implications. Fertil Steril 2019;112:510–9.
7. Huang L, Bogale B, Tang Y, Lu S, Xie XS, Racowsky C. Noninvasive preimplantation genetic testing for aneuploidy in spent medium may be more reliable than trophectoderm biopsy. Proc Natl Acad Sci USA 2019;116:14105–12.
8. Lledó B, Morales R, Ortiz JA, Rodriguez-Arnedo A, Ten J, Castillo JC, et al. Consistent results of non-invasive pre-implantation genetic testing for aneuploidy (niPGT-A) of human embryos using two different techniques for chromosomal analysis. Reprod Biomed Online. In press.
9. Rubio C, Navarro-Sánchez L, García-Pascual CM, Ocali O, Cimadomo D, Venier W, et al. Multicenter prospective study of concordance between embryo cell-free DNA and trophectoderm biopsies from 1,301 human blastocysts. Am J Obstet Gynecol 2020;223:751.e1–13.
10. Xu J, Fang R, Chen L, Chen D, Xiao JP, Yang W, et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. Proc Natl Acad Sci USA 2016;113:11907–12.
11. Yin B, Zhang H, Xie J, Yao Y, Wei Y, Zhang C, et al. Validation of preimplantation genetic tests for aneuploidy (PGT-A) with DNA from spent culture media (SCM): concordance assessment and implication. Reprod Biol Endocrinol. In press.
12. Hardy K, Spanos S, Becker D, Iannelli P, Winston RM, Stark J. From cell death to embryo arrest: mathematical models of human preimplantation embryo development. Proc Natl Acad Sci USA 2001;98:1655–60.
13. Kuznyetsov V, Madjunkova S, Abramov R, Antes R, Ibarrientos Z, Motamedi G, et al. Minimally invasive cell-free human embryo aneuploidy testing (miPGT-A) utilizing combined spent embryo culture medium and blastocoel fluid—towards development of a clinical assay. Sci Rep 2020;10:1–2.
14. Neal SA, Morin SJ, Franasiak JM, Goodman LR, Juneau CR, Forman EJ, et al. Preimplantation genetic testing for aneuploidy is cost-effective, shortens treatment time, and reduces the risk of failed embryo transfer and clinical miscarriage. Fertil Steril 2018;110:896–904.
15. Somigliana E, Busnelli A, Paffoni A, Vigano P, Riccaboni A, Rubio C, Capalbo A. Cost-effectiveness of preimplantation genetic testing for aneuploidies. Fertil Steril 2019;111:1169–76.
16. Zhang WY, von Versen-Höynck F, Kapphahn KI, Fleischmann RR, Zhao Q, Baker VL. Maternal and neonatal outcomes associated with trophectoderm biopsy. Fertil Steril 2019;112:283–90.
17. Makhijani R, Bartels CB, Godiwala P, Bartolucci A, DiLuigi A, Nulsen J, et al. Impact of trophectoderm biopsy on obstetric and perinatal outcomes following frozen–thawed embryo transfer cycles. Hum Reprod 2021;36:340–8.
18. Meyers J, Laliberte J, Choudhary K, Yasuyama N, Sifuentes CJ, Xianget W, et al. Assessment of cell-free DNA from embryo spent media and its effects on non-invasive PGT-A concordance to conventional PGT-A and calculated copy number noise. Fertil Steril 2020;114:e422.
19. Kaye L, Anspach E, Bartolucci A, Nulsen J, Benadiva C, Engmann L. Pregnancy rates for single embryo transfer (SET) of day 5 and day 6 blastocysts after cryopreservation by vitrification and slow freeze. J Assist Reprod Genet 2017;34:913–9.
20. Tiegs AW, Sun L, Patounakis G, Scott RT Jr. Worth the wait? Day 7 blastocysts have lower euploidy rates but similar sustained implantation rates as day 5 and day 6 blastocysts. Hum Reprod 2019;34:1632–9.
21. Bourdon M, Pocate-Cheriet K, de Bantel AF, Grzegorczyk-Martin V, Hoffet AA, Arboet E, et al. Day 5 versus day 6 blastocyst transfers: a systematic review and meta-analysis of clinical outcomes. Hum Reprod 2019;34:1948–64.
22. Cimadomo D, Soscia D, Vaiarelli A, Maggiulli R, Capalbo A, Ubaldi FP, et al. Looking past the appearance: a comprehensive description of the clinical contribution of poor-quality blastocysts to increase live birth rates during cycles with aneuploidy testing. Hum Reprod 2019;34:1206–14.
23. Sacchi L, Albani E, Cesana A, Smeraldi A, Parini V, Fabiani M, et al. Preimplantation genetic testing for aneuploidy improves clinical, gestational, and neonatal outcomes in advanced maternal age patients without compromising cumulative live-birth rate. J Assist Reprod Genet 2019;36:2493–504.
24. Rubino P, Tapia L, Ruiz de Assin R, Mazmanian K, Guan L, Dearden L, et al. Trophectoderm biopsy protocols can affect clinical outcomes: time to focus on the blastocyst biopsy technique. Fertil Steril 2020;113:981–9.
25. Friedenthal J, Maxwell SM, Tiegs AW, Besser AG, McCaffrey C, Munné S, et al. Clinical error rates of next generation sequencing and array comparative genomic hybridization with single thawed euploid embryo transfer. Eur J Med Genet 2020;63:103852.
26. Shear MA, Vaugham DA, Modest AM, Seidler EA, Leung AQ, Hacker MR, et al. Blast from the past: is morphology useful in PGT-A tested and untested frozen embryo transfers? Reprod Biomed Online 2020;41:981–9.
27. Brouillet S, Martinez G, Coutton C, Hamamah S. Is cell-free DNA in spent embryo culture medium an alternative to embryo biopsy for preimplantation genetic testing? A systematic review. Reprod Biomed Online 2020;40:779–96.
28. Hammond ER, McGillvary BC, Wicker SM, Peek JC, Shelling SN, Stone P, et al. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. Fertil Steril 2017;107:220–8.
29. Li P, Song Z, Yao Y, Huang T, Mao R, Huang J, et al. Preimplantation genetic screening with spent culture medium/blastocoel fluid for in vitro fertilization. Sci Rep 2018;8:9275.
30. Capalbo A, Ubaldi FM, Rienzi L, Scott R, Treff N. Detecting mosaicism in trophectoderm biopsies: current challenges and future possibilities. Hum Reprod 2017;32:492–8.
31. Leaver M, Wells D. Non-invasive preimplantation genetic testing (niPGT): the next revolution in reproductive genetics? Hum Reprod Update 2020;26:16–42.
32. Popovic M, Dhaenens L, Boel A, Menten B, Heindryckx B. Chromosomal mosaicism in human blastocysts: the ultimate diagnostic dilemma. Hum Reprod Update 2020;26:313–34.
33. Paulson RJ. Preimplantation genetic screening: What is the clinical efficiency? Fertil Steril 2017;108:228–30.
34. Greco E, Minasi MG, Fiorentino F. Healthy babies after intrauterine transfer of mosaic aneuploid blastocysts. N Engl J Med 2015;373:2089–90.
35. Patrizio P, Shoham G, Shoham Z, Leong M, Barad DH, Gleicher N. Worldwide live births following the transfer of chromosomally "Abnormal" embryos after PGT/A: results of a worldwide web-based survey. J Assist Reprod Genet 2019;36:1599–607.
36. Kuznyetsov V, Madjunkova S, Antes R, Abramov R, Motamedi G, et al. Evaluation of a novel non-invasive preimplantation genetic screening approach. PLoS One 2018;13:e0197262.
37. Chen J, Jia L, Li T, Guo Y, He S, Zhang Z, et al. Diagnostic efficiency of blastocyst culture medium in noninvasive preimplantation genetic testing (niPGT). F S Rep. In press.
38. Magli MC, Albanese C, Crippa A, Tabanelli C, Ferraretti AP, Gianaroli L. Deoxyribonucleic acid detection in blastocoelic fluid: a new predictor of embryo ploidy and viable pregnancy. Fertil Steril 2019;111:77–85.

39. Bolton H, Graham SJL, Van der Aa N, Kumar P, Theunis K, Gallardo EF, et al. Mouse model of chromosome mosaicism reveals lineage-specific depletion of aneuploid cells and normal developmental potential. Nat Commun 2016; 7:11165.
40. Zhang Y, Li N, Wang L, Huiying S, Minyue M, Xiaofei X, et al. Molecular analysis of DNA in blastocoele fluid using next-generation sequencing. J Assist Reprod Genet 2016;33:637–45.
41. Xu XP, Gan HY, Li FX, Tian Q, Zhang J, Liang RL, et al. A method to quantify cell-free fetal DNA fraction in maternal plasma using next generation sequencing: its application in non-invasive prenatal chromosomal aneuploidy detection. PLoS One 2016;11:e0146997.
42. Taglauera E, Wilkins-Haugh L, Bianchi D. Review: cell-free fetal DNA in the maternal circulation as an indication of placental health and disease. Placenta 2014;35:S64–8.
43. Munne S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, et al. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. Fertil Steril 2019;112:1071–9.
44. Scriven PN, Bossuyt PM. Diagnostic accuracy: theoretical models for preimplantation genetic testing of a single nucleus using the fluorescence in situ hybridization technique. Hum Reprod 2010;25:2622–8.
45. Mastenbroek S, Twisk M, van der Veen F, Repping S. Preimplantation genetic screening: a systematic review and meta-analysis of RCTs. Hum Reprod Update 2011;17:454–66.
46. Gleicher N, Vidali A, Braverman J, Kushnir VA, Barad DH, Hudson C, et al. Accuracy of preimplantation genetic screening (PGS) is compromised by degree of mosaicism of human embryos. Reprod Biol Endocrinol 2016;14:1–7.
47. Munne S, Spinella F, Grifo J, Zhang J, Beltran MP, Fragouli E, et al. Clinical outcomes after the transfer of blastocysts characterized as mosaic by high resolution next generation sequencing—further insights. Eur J Med Genet 2020;63:103741.
48. Gleicher N, Metzger J, Croft G, Kushnir VA, Albertini DF, Barad DH. A single trophectoderm biopsy at blastocyst stage is mathematically unable to determine embryo ploidy accurately enough for clinical use. Reprod Biol Endocrinol 2017;15:1–8.
49. Tocci A. The unknown human trophectoderm: implication for biopsy at the blastocyst stage. J Assist Reprod Genet 2020;37:2699–711.
50. Barad DH, Darmon SK, Kushnir VA, Albertini DF, Gleicher N. Impact of preimplantation genetic screening on donor oocyte-recipient cycles in the United States. Am J Obstet Gynecol 2017;217:576.e1–8.
51. Santos MA, Teklenburg G, Macklon NS, Van Opstal D, Schuring-Blom GH, Krijtenburg PJ, et al. The fate of the mosaic embryo: chromosomal constitution and development of day 4, 5 and 8 human embryos. Hum Reprod 2010; 25:1916–26.
52. Singla S, Iwamoto-Stohl LK, Zhu M, Zernicka-Goetz M. Autophagy-mediated apoptosis eliminates aneuploid cells in a mouse model of chromosome mosaicism. Nat Commun 2020;11:1–5.
53. Yang M, Rito T, Naftaly J, Hu J, Albertini DF, Barad DH, et al. Self-correction of mosaicism in human self-organizing gastruloids as potential explanation for normal births after transfer of chromosomal-abnormal embryos. Fertil Steril 2020;114:e14–5.
54. Munne S, Alikani M, Ribustello L, Colls P, Martinez-Ortiz PA, McCulloh DH, et al. Euploidy rates in donor egg cycles significantly differ between fertility centers. Hum Reprod 2017;32:743–9.
55. Tiegs AW, Tao X, Whitehead CV, Neal SA, Osman EK, Kim JG, et al. Does preimplantation genetic testing for aneuploidy (PGT-A) harm embryos? no—a multi-center, prospective, blinded, non-selection study evaluating the predictive value of an aneuploid diagnosis and impact of biopsy. Fertil Steril 2019;112:e31.
56. Tran D. Class action launched against Monash IVF over non-invasive embryo testing. Available at: https://www.msn.com/en-au/news/australia/class-action-launched-against-monash-ivf-over-non-invasive-embryo-testing/ar-BB1capup. Accessed February 17, 2021.
57. Scott RT Jr, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, et al. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. Fertil Steril 2013;100:697–703.
58. Forman EJ, Hong KH, Ferry KM, Tao X, Taylor D, Levy B, et al. In vitro fertilization with single euploid blastocyst transfer: a randomized controlled trial. Fertil Steril 2013;100:100–7.
59. Scott RT Jr, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. Fertil Steril 2013; 100:624–30.
60. Palini S, Galluzzi L, De Stefani S, Bianchi M, Wells D, Magnani M, et al. Genomic DNA in human blastocoele fluid. Reprod Biomed Online 2013; 26:603–10.
61. Hanson BM, Tao X, Kim JG, Herlihy NS, Klimczak, Tiegs AM, et al. Noninvasive preimplantation genetic testing for aneuploidy exhibits high rates of DNA amplification failure and lacks consistency with PGT-A using trophectoderm biopsy. Fertil Steril. In press.
62. Liu WQ, Liu JQ, Du HZ, Ling JW, Sun XF, Chen DJ. Non-invasive preimplantation aneuploidy screening and diagnosis of beta thalassemia IVSII654 mutation using spent embryo culture medium. Ann Med 2017;49:319–28.
63. Werneck A. Lawsuit against monash IVF over embryo testing method. Available at: https://www.bionews.org.uk/page_154143. Accessed January 18, 2021.